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IN VITRO COMPARISON OF BIOFILM METABOLIC ACTIVITY ON ALIGNERS FROM FULL-SERVICE AND IN-OFFICE FABRICATED PLATES

ANÁLISE DA FORMAÇÃO DE BIOFILME MULTIESPÉCIE EM ALINHADORES ORTODÔNTICOS FABRICADOS PELOS MÉTODOS *FULL-SERVICE* E *IN-OFFICE*

COMPARACIÓN IN VITRO DE LA ACTIVIDAD METABÓLICA DEL BIOFILM EN ALINEADORES DE ORTODONCIA MEDIANTE LOS MÉTODOS FULL-SERVICE Y IN-OFFICE

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ABSTRACT

Objective: To compare the metabolic activity of a multispecies subgingival biofilm formed on clear aligners produced by full-service manufacturers and thermoformed plates used for in-office aligner fabrication, according to material composition and manufacturing workflow. Methods: Ten types of thermoformed aligners were evaluated: four from full-service systems (Invisalign®, ClearCorrect®, NewAligner®, and SouSmile®) and six commonly produced for in-office treatment protocol. The in-office aligners were thermoformed on 3D printed models produced by either stereolithography/liquid crystal display (SLA/LCD) or fused deposition modeling (FDM). Rectangular specimens (5 × 8 × 0.75 mm) were sectioned from the buccal surface of maxillary central incisor region (n = 8 per group) and exposed for 7 days to a complex multispecies subgingival biofilm. Biofilm metabolic activity was quantified using 2,3,5-triphenyltetrazolium chloride (TTC) reduction and spectrophotometric analysis. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. The level of significance was 5%. Results: The results showed statistically significant differences between and within groups. However, no statistically significant differences were observed between the 3D model printing methods used for in-office aligner fabrication. Conclusion: The groups with the highest and lowest metabolic activity were both within full-service systems. The fabrication methodology had no significant influence on biofilm metabolic activity, nor did the 3D printing method used for in-office model production.

KEYWORDS: *Orthodontic appliance. Clear aligner. Bacterial biofilm. Biofilm metabolism.*

RESUMO

Objetivo: Comparar a atividade metabólica de biofilme subgengival multiespécies, formado sobre alinhadores transparentes produzidos por empresas especializadas, no método *full-service* e placas termoformadas utilizadas para confecção de alinhadores em consultório, no método *in-office*, considerando a composição do material e o fluxo de fabricação. Métodos: Foram avaliados dez tipos de alinhadores termoformados: quatro advindos do método *full-service* de quatro diferentes empresas (Invisalign®, ClearCorrect®, NewAligner® e SouSmile®) e seis comumente produzidos para protocolos de tratamento em consultório (sistema *in-office*).

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Os alinhadores *in-office* foram termoformados sobre modelos impressos em 3D, produzidos por estereolitografia/visualização por cristal líquido (SLA/LCD) ou modelagem por deposição fundida (FDM). Dos alinhadores obtidos após termoplastificação, foram seccionados espécimes retangulares (5 × 8 × 0,75 mm) da superfície vestibular da região do incisivo central superior (n = 8 por grupo) e expostos por 7 dias a um biofilme subgengival complexo, multiespécies. A atividade metabólica do biofilme foi quantificada por redução de cloreto de 2,3,5-trifeniltetrazólio (TTC) e análise espectrofotométrica. Os dados foram analisados por ANOVA one-way, seguida pelo teste post hoc de Tukey. O nível de significância adotado foi de 5%. Resultados: Os resultados mostraram diferenças estatisticamente significativas inter e intra-grupos. Entretanto, não foram observadas diferenças estatisticamente significativas entre os métodos de impressão 3D utilizados durante a confecção dos alinhadores *in-office*. Conclusão: Os grupos com maior e menor atividade metabólica estavam ambos dentro dos sistemas *full-service*. A metodologia de fabricação não influenciou significativamente a atividade metabólica do biofilme, assim como o método de impressão 3D utilizado para a produção dos modelos em consultório.

PALAVRAS-CHAVE: Aparelho ortodôntico. Alinhador transparente. Biofilme bacteriano. Metabolismo do biofilme.

RESUMEN
Objetivo: Comparar la actividad metabólica de un biofilm subgingival multiespecies formado sobre placas transparentes producidas por fabricantes de servicio completo y placas termomoldeadas utilizadas para la fabricación de alineadores en consultorio, según la composición del material y el flujo de fabricación. Métodos: Se evaluaron diez tipos de alineadores termomoldeados: cuatro de sistemas de servicio completo (Invisalign®, ClearCorrect®, NewAligner® y SouSmile®) y seis comúnmente producidos para protocolos de tratamiento en consultorio. Los alineadores de consultorio fueron termomoldeados sobre modelos impresos en 3D, producidos mediante estereolitografía/visualización por cristal líquido (SLA/LCD) o modelado por deposición fundida (FDM). Se seccionaron especímenes rectangulares (5 × 8 × 0,75 mm) de la superficie vestibular de la región del incisivo central superior (n = 8 por grupo) y se expusieron durante 7 días a un biofilm subgingival complejo y multiespecies. La actividad metabólica del biofilm se cuantificó mediante reducción de cloruro de 2,3,5-trifeniltetrazolio (TTC) y análisis espectrofotométrico. Los datos se analizaron mediante ANOVA de un factor, seguido de la prueba post hoc de Tukey. Se adoptó un nivel de significancia del 5%. Resultados: Los resultados mostraron diferencias estadísticamente significativas entre y dentro de los grupos. Sin embargo, no se observaron diferencias estadísticamente significativas entre los métodos de impresión 3D utilizados para la fabricación de alineadores en consultorio. Conclusión: Los grupos con mayor y menor actividad metabólica pertenecían ambos a sistemas de servicio completo. La metodología de fabricación no tuvo una influencia significativa sobre la actividad metabólica del biofilm, al igual que el método de impresión 3D utilizado para la producción de modelos en consultorio.

PALABRAS CLAVE: *Aparato ortodóntico, placa transparente para alineadores, biofilm bacteriano, metabolismo del biofilm.*

INTRODUCTION

The increasing demand for orthodontic treatment with clear aligners is primarily driven by their esthetic appeal, comfort, predictable force delivery, and removability compared with fixed appliances[1,2,3]. Since their introduction in the late 1990s, aligner systems have evolved substantially, incorporating digital workflows based on computer-aided design and computer-aided

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manufacturing (CAD/CAM) and additive manufacturing technologies to produce customized orthodontic appliances from thermoplastic polymers[4].

A key advantage of clear aligners is the facilitation of oral hygiene maintenance, owing to the absence of plaque-retentive elements and the possibility of removal during routine toothbrushing. Fixed orthodontic appliances are recognized as plaque-retentive structures, increasing the risk of enamel demineralization, dental caries, gingivitis, and periodontal disease[5]. Although aligners are removable and lack brackets or wires, they present non-shedding intraoral surfaces in prolonged contact with teeth and gingival tissues, which may favor microbial adhesion and biofilm maturation. Therefore, the impact of prolonged daily use and extended use of retention plates on biofilm formation warrants further investigation.

Periodontal disease is initiated and sustained by a complex subgingival biofilm dominated by Gram-negative, anaerobic, proteolytic microorganisms. The ecological balance of this biofilm is influenced by multiple factors, including surface characteristics of intraoral materials, local environmental conditions, host response, and oral hygiene practices[6,7,8]. Previous studies have demonstrated that even patients treated exclusively with clear aligners may experience qualitative and quantitative changes in the subgingival microbiota within weeks of treatment initiation, indicating that aligners are not biologically inert with respect to microbial colonization. Recent clinical and in vitro investigations suggest that aligner therapy can promote microbial adhesion on thermoplastic surfaces and alter subgingival biofilm composition over time, reinforcing the need for material-specific biological assessments[9].

Biofilm formation on orthodontic materials is strongly modulated by surface-related properties, including roughness, surface free energy, wettability, and chemical composition. Indented and sheltered areas of aligners, such as attachment reliefs, cusp coverage, and mechanically stressed regions, promote greater plaque accumulation than flat surfaces. Consequently, both aligner design and intrinsic material characteristics play a decisive role in microbial adhesion and subsequent biofilm development[10,11].

Currently, aligners are produced via two main workflows: full-service commercial systems and in-office fabrication by orthodontists. Full-service aligners are typically manufactured from proprietary multilayer polymer blends. In contrast, in-office aligners are commonly thermoformed from polyethylene terephthalate glycol (PET-G) plates over three-dimensionally printed working models[12-14]. These models may be fabricated using stereolithography/liquid crystal display (SLA/LCD) or fused deposition modeling (FDM) additive manufacturing techniques. SLA/LCD employs laser-based photopolymerization of resin layers, whereas FDM extrudes heated thermoplastic filaments layer by layer to produce 3D structures[15,16,17].

From a materials science perspective, aligners differ substantially in polymer architecture, viscoelastic behavior, and water absorption, which influence surface stability and interaction with

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the oral environment, potentially affecting microbial adhesion and biofilm formation[12]. Most previous microbiological studies have evaluated a limited number of materials or brands, often using.

Simplified mono- or dual-species biofilm models. No study to date has comprehensively compared biofilm formation on a wide range of full-service aligners and in-office thermoformed plates using a complex multispecies subgingival biofilm[18].

Therefore, the aim of this in vitro study was to compare the metabolic activity of a complex multispecies subgingival biofilm formed on clear aligners produced by full-service manufacturers and by in-office fabrication workflows. The null hypotheses were: (1) no difference in biofilm metabolic activity among aligners from different full-service and in-office systems; (2) the type of 3D printing technology used for model fabrication (SLA/LCD vs FDM) does not influence biofilm metabolic activity.

MATERIAL AND METHODS

Study Design

This in vitro study evaluated biofilm formation on orthodontic clear aligners manufactured from different thermoplastic materials and produced through distinct fabrication workflows under standardized laboratory conditions.

Materials and manufacturing techniques were selected based on their widespread clinical use in both full-service and in-office aligner systems. The experimental design and analytical procedures were established prior to specimen preparation. As this study was conducted entirely in vitro, approval from an ethics committee was not required.

Sample Size and Experimental Groups

Sample size was determined according to methodological standards commonly adopted in in vitro biofilm studies, in which six to ten specimens per group are generally considered sufficient to detect biologically relevant differences in microbial growth and metabolic activity. Accordingly, eight specimens were allocated to each experimental group[19].

Ten types of ready-to-use aligners were evaluated (Table 1). Four aligners were obtained from full-service manufacturers—Invisalign®, ClearCorrect®, NewAligner®, and SouSmile®—and were designated as Group A. Six in-office aligners were fabricated from PET-G sheets supplied by Duran®, Forestadent®, Essix ACE®, Pro-Aligner®, Bio-Art®, and Orthomundi®, and were designated as Group B.

Therefore, the total sample size was 80 specimens, 8 per group.

Group B was further subdivided according to the 3D-printing technology used to fabricate the working models:

- Group B1: aligners thermoformed on resin models produced by stereolithography/liquid

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crystal display (SLA/LCD) printers (n = 4);

- Group B2: aligners thermoformed on filament models produced by fused deposition

- modeling (FDM) printers (n = 4).

The Group B aligners were thermoformed on uncrowded resin models using a Bio-Art vacuum laminator (Bio-Art, São Carlos, Brazil). The full-service aligners (Group A) were supplied directly by the manufacturers and required no additional processing.

Specimen Preparation

Specimens were obtained from the buccal surface of the maxillary central incisor region of each aligner. This region was selected because of its relatively flat morphology, allowing the preparation of standardized specimens. Rectangular samples measuring 5 mm × 8 mm with a thickness of 0.75 mm were sectioned from each aligner. Prior to microbiological testing, all specimens were cleaned, sterilized, and handled under aseptic conditions.

Biofilm Formation

A total of 33 bacterial species were cultured to establish a mature and complex multispecies biofilm model (Figure 1). Among them, *Porphyromonas gingivalis* and *Tannerella forsythia* are recognized as key periodontal pathogens strongly associated with periodontal tissue destruction. Several microorganisms involved in biofilm maturation and the establishment of pathogenic microbial communities were also included, such as *Prevotella intermedia*, *Campylobacter showae*, *Campylobacter gracilis*, *Fusobacterium nucleatum subsp. vincentii*, *Fusobacterium nucleatum subsp. polymorphum*, *Fusobacterium periodonticum*, *Parvimonas micra*, and *Eubacterium nodatum*. Additional species commonly found in oral biofilms, including *Aggregatibacter actinomycetemcomitans*, *Capnocytophaga spp.*, *Streptococcus sanguinis*, *Streptococcus oralis*, *Streptococcus gordonii*, *Veillonella parvula*, and *Actinomyces spp.*, were incorporated to reproduce the diversity of microbial interactions occurring in the oral environment. The model also included cariogenic bacteria, such as *Streptococcus mutans*, together with established periodontal pathogens, thereby representing both cariogenic and periodontopathogenic challenges. The coexistence of early colonizers, biofilm-bridging organisms, cariogenic species, and highly pathogenic periodontal microorganisms provides a biologically complex and clinically relevant model that more closely resembles the polymicrobial biofilms found in vivo than simplified mono- or dual-species systems. Therefore, the metabolic activity measured in the present study reflects the behavior of a mature and ecologically diverse microbial community rather than that of isolated bacterial species.

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The microorganisms were initially cultured on tryptic soy agar supplemented with 5% sheep blood under anaerobic conditions (85% N_2, 10% CO_2, and 5% H_2).

Porphyromonas gingivalis and *Prevotella intermedia* were cultured on tryptic soy agar supplemented with yeast extract, 1% hemin, 5% menadione, and 5% sheep blood.
Tannerella forsythia was cultured on tryptic soy agar supplemented with yeast extract, 1% hemin, 5% menadione, 5% sheep blood, and 1% N-acetylmuramic acid.

After 48 hours of incubation, all bacterial strains were transferred to brain heart infusion (BHI) broth supplemented with 1% hemin. Following an additional 24 hours of incubation, the optical density was adjusted to 0.1 at 600 nm, corresponding to approximately 10^8 cells/mL for each species. To prepare the final inoculum, individual bacterial suspensions were diluted to obtain a mixed suspension containing 10^4 cells/mL of each species. This multispecies inoculum was used to develop subgingival biofilms in 96-well microplates.

Specimens were positioned vertically within the wells and exposed to the inoculum for seven days. The culture medium was renewed on the third day to maintain adequate nutrient availability and support biofilm development.

Each specimen was placed individually in the wells without adhesives or mechanical fixation. The dimensions of the wells were sufficient to maintain the specimens in a stable vertical position throughout the experimental period, preventing displacement and unintended contact with the well walls. This configuration ensured standardized exposure of all specimen surfaces to the bacterial suspension and culture medium. Biofilm development followed the protocols previously described by Miranda *et al.*[20] and Pingueiro *et al.*[21]

Microbiological analysis – Metabolic Activity of the Biofilm

Biofilm metabolic activity was quantified using the 2,3,5-triphenyltetrazolium chloride (TTC) reduction assay followed by spectrophotometric analysis.

TTC is a colorless substrate that is enzymatically reduced by metabolically active bacterial cells through the action of dehydrogenase enzymes, producing red-colored 1,3,5-triphenylformazan (TPF). The resulting color change can be quantified spectrophotometrically and serves as an indirect indicator of biofilm metabolic activity.

For this analysis, three specimens were randomly selected from each experimental group and replicated. Samples were washed twice with a washing solution and transferred to new microplates containing 200 µL of fresh BHI broth supplemented with 1% hemin and 0.1% TTC. The specimens were incubated under anaerobic conditions at 37°C for 6–8 hours. Subsequently, TTC reduction was measured at 485 nm using a fluorescence spectrophotometer[19].

All analyses were performed by a blinded examiner who was unaware of group allocation.

Statistical Analysis

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Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Statistical significance was established at $p \leq 0.05$. All analyses were performed using Biostat software.

RESULTS

The results are presented in Figures 2–5. Multiple comparisons were performed to evaluate biofilm metabolic activity among all tested aligner materials.

Among the full-service aligners (Group A), Invisalign® and NewAligner® exhibited the lowest levels of biofilm metabolic activity (mean 0.391 and 0.338) and differed significantly from SouSmile®, which showed the highest metabolic activity (mean 1.261). ClearCorrect® demonstrated intermediate values (mean 0.794) and did not differ significantly from the remaining materials (Figure 2).

Although statistically significant differences were detected among specific in-office aligner materials, the overall range of biofilm metabolic activity values was relatively narrow, varying from 0.376 (Forestadent®-B1) to 0.707 (Essix®-B2). Notably, materials assigned to different statistical groups often exhibited similar mean values, such as Orthomundi®-B1 (0.609) and Orthomundi®-B2 (0.584). Therefore, no consistent pattern associated with the aligner material or fabrication method was observed. Overall, the in-office aligners demonstrated comparable biofilm metabolic activity (Figure 3).

To further investigate the influence of the 3D-printing technology used for model fabrication, subgroups B1 (SLA/LCD) and B2 (FDM) were compared separately. No statistically significant differences in biofilm metabolic activity were observed between the two printing methods (Figure 4).

When all aligner systems were analyzed collectively, several statistically significant differences were identified among brands (Figure 5). Nevertheless, the materials exhibiting the highest and lowest biofilm metabolic activity remained unchanged. SouSmile® demonstrated the highest metabolic activity, whereas NewAligner® exhibited the lowest.

Among the in-office aligners, Pro-Aligner® (B1 and B2), Forestadent® (B1), and Duran® (B2) demonstrated significantly lower metabolic activity (from 0.338 to 0.472) than SouSmile® (mean 1.261) and values comparable to those observed for NewAligner® ($p \leq 0.05$). The remaining materials showed intermediate behavior, with no statistically significant differences relative to either extreme.

DISCUSSION

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Biofilm formation is a dynamic and multifactorial process that plays a central role in the initiation and progression of periodontal diseases. Within the oral cavity, microorganisms colonize both natural and artificial surfaces, forming highly organized microbial communities embedded within an extracellular polymeric matrix composed of polysaccharides, proteins, and nucleic acids[22]. Orthodontic appliances, including clear aligners, provide additional non-shedding surfaces that may facilitate microbial adhesion, particularly in areas that are less accessible to mechanical cleaning[23]. Therefore, understanding the interaction between aligner materials and oral biofilms is essential for evaluating the biological implications of aligner therapy.

Subgingival biofilm is recognized as the primary etiological factor in periodontal disease. The microbial complexes described by Socransky *et al.*[24,25] established a biological framework linking specific bacterial communities to periodontal health and disease. In the present study, a complex multispecies subgingival biofilm composed of 33 bacterial species was employed, providing a more clinically relevant model than the simplified mono- or dual-species biofilms frequently used in laboratory investigations. Consequently, the present findings may offer a more realistic representation of the microbial interactions occurring on aligner surfaces under clinical conditions.

One of the most important findings of this study is that biofilm metabolic activity was not consistently associated with the manufacturing workflow itself (full-service versus in-office fabrication). Although statistically significant differences were observed among individual full-service systems, these differences were not uniformly distributed across the group. Notably, only one full-service aligner system exhibited substantially greater biofilm metabolic activity, whereas the remaining full-service aligners demonstrated values comparable to, or lower than, several in-office fabricated aligners (Figure 2, 3 and 4). These findings suggest that biofilm formation cannot be explained solely by the manufacturing workflow. Instead, material-specific characteristics and processing-related factors intrinsic to each aligner system are likely to play a more relevant role in determining microbial adhesion and biofilm development.

The variability observed among full-service aligners may be partially attributed to differences in polymer composition, multilayer architecture, manufacturing processes, or surface properties. However, this interpretation remains speculative because detailed information regarding material composition is generally not disclosed by manufacturers for proprietary reasons. Additional studies combining microbiological analyses with comprehensive material characterization are therefore warranted.

The present results are consistent with previous investigations demonstrating that clear aligners may promote microbial colonization on thermoplastic surfaces despite their removable nature. Although aligners can be removed for oral hygiene procedures, their prolonged daily wear and intimate contact with teeth and gingival tissues may create favorable ecological conditions for microbial adhesion and biofilm maturation[9]. Such observations reinforce the importance of

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evaluating the biological performance of different aligner materials rather than assuming equivalent behavior among commercially available systems.

All in-office aligners evaluated in this study were fabricated from PET-G-based materials, which may explain the relatively homogeneous metabolic activity observed within this group. The limited differences identified among brands may reflect subtle variations in polymer formulation, including the incorporation of copolyester components or differences in manufacturing processes. Nevertheless, because manufacturers do not fully disclose the chemical composition of their materials, the present study cannot determine whether these factors directly influenced biofilm formation[11,25].

Surface characteristics may also contribute to the observed differences in microbial behavior. Previous studies have demonstrated that increased surface roughness promotes bacterial adhesion by creating protected microenvironments that facilitate irreversible biofilm establishment[8]. Although surface roughness was not evaluated in the present investigation, its potential influence cannot be excluded and should be addressed in future studies. Biofilm formation on orthodontic aligners is strongly influenced by the physicochemical properties of the thermoplastic materials used. These include polymer composition, viscoelastic behavior, water absorption, surface free energy, wettability, and surface roughness, all of which can modulate bacterial adhesion and subsequent biofilm maturation. Multilayer polymers used in full-service aligners may exhibit different hydrophobicity and surface charge profiles compared to PET-G-based plates used for in-office fabrication, which can alter initial bacterial attachment and metabolic activity[18]. Furthermore, thermoforming parameters, such as temperature and pressure, may affect the final surface microtopography, thereby creating niches favorable for microbial colonization. Understanding these physicochemical characteristics is essential to predict material-specific biofilm development and to inform selection of aligners with lower microbial retention potential.

Regarding the in-office workflow, the type of 3D-printing technology used for model fabrication—resin-based SLA/LCD or filament-based FDM—did not significantly affect biofilm metabolic activity (Figure 3). Although FDM-produced models are frequently associated with greater surface irregularities, these characteristics did not appear to translate into biologically meaningful differences in the thermoformed aligners. This finding suggests that, when appropriate thermoforming protocols are employed, both manufacturing approaches can generate aligner surfaces with comparable susceptibility to biofilm development.

Several limitations should be acknowledged. Surface roughness, wettability, surface free energy, and detailed chemical composition were not directly evaluated. Furthermore, the in vitro design cannot fully reproduce the complexity of the oral environment, including salivary flow, dietary influences, mechanical wear, host factors, and individual oral hygiene practices. Therefore, caution should be exercised when extrapolating these findings to clinical conditions.

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Future investigations should prioritize in vivo validation, comprehensive surface characterization, and detailed microbial profiling. In addition, growing interest has been directed toward the incorporation of antimicrobial agents into aligner materials as a strategy to reduce bacterial colonization[26,27]. Further research exploring surface modifications and antimicrobial technologies may contribute to the development of aligners with improved biological and hygienic performance.

CONCLUSION

Based on the methodology employed and the results obtained, it can be concluded that the aligners exhibiting the highest and lowest biofilm metabolic activity were both full-service systems. Neither the fabrication workflow nor the 3D printing technology used for in-office model production significantly influenced biofilm metabolic activity. From a clinical perspective, these findings indicate that full-service and in-office aligner systems present comparable susceptibility to biofilm development, allowing treatment decisions to be guided primarily by clinical, logistical, and economic considerations rather than by the manufacturing workflow itself.

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